



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>A61K 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/08845</b>  <b>(43) International Publication Date:</b> 13 May 1993 (13.05.93)
<b>(21) International Application Number:</b> PCT/US92/09626 <b>(22) International Filing Date:</b> 5 November 1992 (05.11.92)  <b>(30) Priority data:</b> 792,146 8 November 1991 (08.11.91) US 855,416 18 March 1992 (18.03.92) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 855,416 (CIP) Filed on 18 March 1992 (18.03.92)  <b>(71) Applicant (for all designated States except US):</b> MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> ROSENBERG, Robert, D. [US/US]; 126 Highland Drive, Jamestown, RI 02835 (US). SIMONS, Michael [US/US]; 115 Grove Street, Chestnut Hill, MA 02167 (US). EDELMAN, Elazer [US/US]; 91 Baxter Road, Brookline, MA 02146 (US). LANGER, Robert, S. [US/US]; 77 Lombard Street, Newton, MA 02159 (US). DEKEYSER, Jean-Luc [BE/BE]; 53, ave. des Gloires Nationales, B-1080 Brussels (BE).		<b>(74) Agent:</b> CAMPBELL, Paula, A.; Testa, Hurwitz & Thibault, Exchange Place, 53 State Street, Boston, MA 02109 (US).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> LOCALIZED OLIGONUCLEOTIDE THERAPY  <b>(57) Abstract</b>  Disclosed is a method for localized application of antisense oligonucleotides, which has been found to be effective in inhibiting expression and translation of a variety of genes. The method utilizes antisense oligonucleotides which are specific for the mRNA transcribed from the gene of interest. The antisense oligonucleotides are applied directly to the desired locus of the cells to be treated, where they hybridize with the mRNA and inhibit expression of the gene. Devices for localized antisense application and methods for making them also are described.		

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## LOCALIZED OLIGONUCLEOTIDE THERAPY

Background of the Invention

This invention relates to a method of delivery of antisense oligonucleotide to a preselected locus in vivo, useful in the treatment of disease.

- 5 In the last several years, it has been demonstrated that oligonucleotides are capable of inhibiting the replication of certain viruses in tissue culture systems. For example, Zamecnik and Stephenson, Proc. Natl. Acad. Sci. U.S.A., 75:280-284 (1978), showed
- 10 oligonucleotide-mediated inhibition of virus replication in tissue culture, using Rous Sarcoma Virus. Zamecnik et al., Proc. Natl. Acad. Sci. U.S.A., 83:4145-4146 (1986), demonstrated inhibition in tissue culture of the HTLV-III virus (now HIV-1) which
- 15 is the etiological agent of AIDS. Oligonucleotides also have been used to suppress expression of selected non-viral genes by blocking translation of the protein encoded by the genes. Goodchild, et al., Arch. Biochem. Biophys., 264:401-409 (1988) report that
- 20 rabbit-globin synthesis can be inhibited by oligonucleotides in a cell-free system. Treatment with antisense c-myb has been shown to block proliferation of human myeloid leukemic cell lines in vitro. G. Anfossi, et al., Proc. Natl. Acad. Sci. USA, 86:3379
- 25 (1989).

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- A drawback to this method is that oligonucleotides are subject to being degraded or inactivated by cellular endogenous nucleases. To counter this problem, some researchers have used modified oligonucleotides, e.g., having altered internucleotide linkages, in which the naturally occurring phosphodiester linkages have been replaced with another linkage. For example, Agrawal et al., Proc Natl. Acad. Sci. U.S.A., 85:7079-7083 (1988) showed increased inhibition in tissue culture of HIV-1 using oligonucleotide phosphoramidates and phosphorothioates. Sarin et al., Proc. Natl. Acad. Sci. U.S.A., 85:7448-7451 (1988) demonstrated increased inhibition of HIV-1 using oligonucleotide methylphosphonates. Agrawal et al., Proc. Natl. Acad. Sci. U.S.A., 86:7790-7794 (1989) showed inhibition of HIV-1 replication in both early-infected and chronically infected cell cultures, using nucleotide sequence-specific oligonucleotide phosphorothioates. Leither et al., Proc. Natl. Acad. Sci. U.S.A., 87:3430-3434 (1990) report inhibition in tissue culture of influenza virus replication by oligonucleotide phosphorothioates.

Oligonucleotides having artificial linkages have been shown to be resistant to degradation in vivo. For example, Shaw et al., in Nucleic Acids Res., 19:747-750 (1991), report that otherwise unmodified

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oligonucleotides become more resistant to nucleases in vivo when they are blocked at the 3' end by certain capping structures and that uncapped oligonucleotide phosphorothioates are not degraded in vivo.

5        While antisense oligonucleotides have been shown to be capable of interfering selectively with protein synthesis, and significant progress has been made on improving their intracellular stability, the problem remains that oligonucleotides must reach their intended  
10 intracellular site of action in the body in order to be effective. Where the intended therapeutic effect is a systemic one, oligonucleotides may be administered systemically. However, when it is necessary or desirable to administer the oligonucleotide to a  
15 specific region within the body, systemic administration typically will be unsatisfactory. This is especially true when the target mRNA is present in normal cells as well as in the target tissue, and when antisense mRNA binding in normal cells induces unwanted  
20 physiological effects. Stated differently, the dosage of antisense oligonucleotide administered systemically that is sufficient to have the desired effect locally may be toxic to the patient.

      An example of a treatment strategy which could  
25 greatly benefit from development of a method of limiting the effect of antisense oligonucleotide to a target tissue is the inhibition of smooth muscle cell proliferation which leads to restenosis following vascular trauma.

30        Smooth muscle cell proliferation is a poorly understood process that plays a major role in a number of pathological states including atherosclerosis and hypertension. It is the leading cause of long-term failure of coronary and peripheral angioplasty as well  
35 as of coronary bypass grafts.

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Vascular smooth muscle cells in adult animals display a well defined phenotype characterized by an abundance of contractile proteins, primarily smooth muscle actin and myosins, as reviewed by S.M. Schwartz, 5 G.R. Campbell, J.H. Campbell, Circ. Res., 58,427 (1986), and a distinct lack of rough endoplasmic reticulum. When subjected to injury in vivo or placed in an in vitro cell culture, adult smooth muscle cells (SMC) undergo a distinct phenotypic change and lose 10 their "differentiated" state. The cells acquire large amounts of endoplasmic reticulum and gain actively synthesizing extracellular matrix. In addition, they begin expressing a number of new proteins including non-muscle myosins and actins, and PDGF A chain, as 15 reported by R.J. Dilley, et al., Atherosclerosis, 63:99 (1987), P. Libby, et al., N. Engl. J. Med., 318:1493 (1988), while the expression of smooth muscle-specific contractile proteins such as smooth muscle myosin heavy chain and alpha actin decline, as shown by M. Kuro-o, 20 et al. J. Biol. Chem., 264:18272 (1989) and A.W. Clowes, et al. J. Cell. Biol., 107:1939 (1988).

A nuclear oncogene c-myb may play an important role in these changes. The oncogene is homologous to the transforming gene of the avian myeloblastosis virus. 25 Although considered originally to be expressed only in hematopoietic cells, c-myb has been shown to be present in chick embryo fibroblasts as well as in proliferating SMCs. C.B. Thompson, et al. Nature, 319:374 (1986); C.F. Reilly, et al. J. Biol Chem., 264:6990 (1989). - 30 The human c-myb gene has been isolated, cloned and sequenced. Majello et al., Proc. Natl. Acad. Sci. USA, 83:9636-9640 (1986). The expression of c-myb is growth-dependent. It is present in a low level in quiescent cells but increases rapidly as cells begin to

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proliferate and peaks near the late G phase of the cell cycle. C.F. Reilly, et al. J. Biol. Chem., 264:6990 (1989). Furthermore, expression of c-myb appears to correlate with the differentiation state of the cell.

- 5 Myeloid erythroleukemia cells have been induced to differentiate and thereby decrease c-myb expression.

Heparin, as well as closely related heparin proteoglycans, can block smooth muscle cell proliferation in vivo as well as in vitro. A.W.

- 10 Clowes, M.J. Karnovsky Nature, 265:625 (1977); C.R. Reilly, et al., J. Cell Physiol., 129, 11 (1986); J.R. Guyton, et al., Circ. Res., 46, 625 (1980); and L.M.S. Fritze, et al., J. Cell. Biol., 100:1041 (1985). This block occurs in a late G phase of the cell cycle and is  
15 associated with a decrease in the level of c-myb (but not that of c-fos or c-myc) expression, (C.F. Reilly, et al., J. Biol. Chem., 264:6990 (1989)), and a partial return of expression of smooth muscle specific contractile proteins. M. Kuro-o, et al., J. Biol.  
20 Chem., 264:18272 (1989) and A.W. Clowes, et al. J. Cell. Biol., 107:1939 (1988). Since c-myb appears to be critically involved in the initiation of proliferation of quiescent smooth muscle cells, heparin may exert its antiproliferative action by its effect on  
25 c-myb. It is an object of the present invention to provide a method for delivery of oligonucleotides to a specific locus in vivo, and thereby to provide localized inhibition of expression of viral genes, oncogenes and genes encoding proteins involved in  
30 disease or other pathologic conditions.

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Summary of the Invention

The present invention relates to a method for inhibiting translation or transcription of a target nucleic acid sequence preferentially at a locus in vivo. The invention involves application directly to the target tissue through a surgical or catheterization procedure of specific oligonucleotides having a nucleotide sequence complementary to at least a portion of the target nucleic acid, i.e., antisense oligonucleotides. The oligonucleotides are preferably antisense sequences specific for the messenger RNA (mRNA) transcribed from the gene whose expression is to be inhibited. The antisense oligonucleotides hybridize with the target mRNA thereby preventing its translation into the encoded protein. Thus, the present method prevents the protein encoded by a selected gene from being expressed. Furthermore, animal experiments have demonstrated dramatic local therapeutic effects in vivo.

The present oligonucleotides preferably are modified to render them resistant to degradation and/or extension by cellular nucleases or other enzymes present in vivo. This can be accomplished by methods known in the art, e.g., by incorporating one or more internal artificial internucleotide linkages, such as replacing the phosphate in the linkage with sulfur, and/or by blocking the 3' end of the oligonucleotide with capping structures. Oligonucleotides of the present invention are preferably between about 14 and 38 nucleotides in length, more preferably between 15 and 30 nucleotides.

The oligonucleotides are applied locally in order to suppress expression of the protein of choice in a circumscribed area. In a preferred embodiment, the



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antisense oligonucleotide is applied to the surface of the tissue at the locus disposed within a biocompatible matrix or carrier. The matrix or carrier can be a hydrogel material such as a poly(propylene oxide-ethylene oxide) gel, e.g., one which is liquid at or below room temperature, and is a gel at body temperature and above. In this embodiment, the oligonucleotides are mixed with the hydrogel material, and the mixture is applied to the desired location during surgery or by catheter. The oligonucleotides also can be applied in solution by liquefying the gel, i.e., by cooling, and are retained at the area of application as the gel solidifies. Other carriers which can be used include, for example, liposomes, microcapsules, erythrocytes and the like. The oligonucleotides also can be applied locally by direct injection, can be released from devices such as implanted stents or catheters, or delivered directly to the site by an infusion pump.

The methods of the present invention are useful in inhibiting the expression of protein encoding genes, as well as regulating non-encoding DNA such as regulatory sequences. Since the antisense oligonucleotides are delivered to a specific defined locus, they can be used in vivo when systemic administration is not possible. For example, systemically administered oligonucleotides may be inactivated by endonucleases rendering them ineffective before they reach their targets. Large doses of the oligonucleotide may be necessary for successful systemic treatment systemically, which may have harmful or toxic effects on the patient. The present method provides a means for treating a large number of specific disorders using oligonucleotide therapy by delivering an antisense sequence to the specific location where it is needed.

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Brief Description of the Drawings

Figures 1A and B are graphs of the cell count for SV-smooth muscle cells (SMC) cells treated with antisense NMMHC (A) and antisense c-myb (B) at various concentrations.

Figures 2A and B are graphs of the cell count for SV40TL-SMC cells, BC3H1 cells, rat aortic SMC, and mouse aortic SMC treated with antisense HNMMHC (A) and c-myb (B).

10 Figure 3A is a graph of the effect on growth of SV-SMC cells treated for different time intervals with antisense c-myb and NMMHC.

Figure 3B is a bar graph showing the effect on SV-SMC cells treated with unmodified antisense c-myb (light bar) and NMMHC (dark bar) for 16 hours and 40 hours after release from growth arrest.

Figure 4 is a bar graph of the results of a c-myb RNA dot blot, showing the amount of mRNA present in SV-SMC cells treated with sense c-myb (S Myb), antisense c-myb (AS Myb) and heparin compared with untreated (control) and growth-arrested (GA) cells.

Figure 5 is a graph showing the release kinetics of oligonucleotides from a Pluronic<sup>TM</sup> 127 gel matrix.

Figure 6 is a graph showing the release kinetics of oligonucleotides from an EVAc matrix.

Figure 7 is a bar graph of the effect on rat arteries of antisense c-myb (AS Myb) using a Pluronic<sup>TM</sup> gel and ethylene vinyl acetate matrix (EVAc) as the delivery systems for application of the oligonucleotides to the injured artery, versus intima/media ratio, a measure of neo-intimal proliferation.

Figure 8 is a bar graph of the effects on rat arteries of antisense c-myb applied using a Pluronic<sup>TM</sup> gel compared to a drug-free gel, a gel containing sense c-myb and an untreated artery.

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Figure 9 is a bar graph showing the effect on rabbit arteries of a mixture of antisense c-myb and human NMMHC (200  $\mu$ M each) on the proliferation of cells in the artery after balloon angioplasty.

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Detailed Description of the Invention

A method for inhibiting expression of protein-encoding genes using antisense oligonucleotides is described. The method is based on the localized  
5 application of the oligonucleotides to a specific site in vivo. The oligonucleotides preferably are applied directly to the target tissue in mixture with an implant or gel, or by direct injection or infusion. In one aspect, the oligonucleotides are treated to render  
10 them resistant in vivo to degradation or alteration by endogenous enzymes.

The Oligonucleotides

The therapeutic approach using antisense  
15 oligonucleotides is based on the principle that the function of a gene can be disrupted by preventing transcription of the gene or translation of the protein encoded by that gene. This can be accomplished by providing an appropriate length oligonucleotide which  
20 is complementary to at least a portion of the messenger RNA (mRNA) transcribed from the gene. The antisense strand hybridizes with the mRNA and targets the mRNA for destruction thereby preventing ribosomal translation, and subsequent protein synthesis.

25 The specificity of antisense oligonucleotides arises from the formation of Watson-Crick base pairing between the heterocyclic bases of the oligonucleotide and complementary bases on the target nucleic acid. For example, a nucleotide sequence sixteen nucleotides  
30 in length will be expected to occur randomly at about every  $4^{16}$ , or  $4 \times 10^9$  nucleotides. Accordingly, such a sequence is expected to occur only once in the human genome. In contrast, a nucleotide sequence of ten nucleotides in length would occur randomly at about

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every  $4^{10}$  or  $1 \times 10^6$  nucleotides. Such a sequence might be present thousands of times in the human genome. Consequently, oligonucleotides of greater length are more specific than oligonucleotides of lesser length and are less likely to induce toxic complications that might result from unwanted hybridization. Therefore, oligonucleotides of the present invention are preferably at least 14 nucleotide bases in length. Oligonucleotides having from about 14 to about 38 bases are preferred, most preferably from about 15 to 30 bases.

The oligonucleotide sequence is selected based on analysis of the sequence of the gene to be inhibited. The gene sequence can be determined, for example, by isolation and sequencing, or if known, through the literature. The sequence of the oligonucleotide is an "antisense" sequence, that is, having a sequence complementary to the coding strand of the molecule. Thus, the sequence of the oligonucleotide is substantially identical to at least a portion of the gene sequence, and is complementary to the mRNA sequence transcribed from the gene. The oligonucleotide therapy can be used to inhibit expression of genes from viruses or other microorganisms that are essential to infection or replication, genes encoding proteins involved in a disease process, or regulatory sequences controlling the expression of proteins involved in disease or other disorder, such as an autoimmune disorder or cardiovascular disease.

Oligonucleotides useful in the present invention can be synthesized by any art-recognized technique for nucleic acid synthesis. The oligonucleotides are preferably synthesized using an automated synthesizer

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such as Model 8700 automated synthesizer (Milligen-Bioscience, Burlington, MA), as described in detail in the Examples below, or an ABI Model 380B using H-phosphonate chemistry on controlled pore glass (CPG).

5 A detailed description of the H-phosphonate approach to synthesizing oligonucleoside phosphorothioates is provided in Agrawal and Tang, Tetrahedron Letters 31:7541-7544 (1990), the teachings of which are hereby incorporated herein by reference. Syntheses of

10 oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters, bridged phosphoramidates and bridge phosphorothioates are known in the art. See, for example, Agrawal and Goodchild, Tetrahedron Letters,

15 28:3539 (1987); Nielsen, et al., Tetrahedron Letters, 29:2911 (1988); Jager et al., Biochemistry, 27:7237 (1988); Uznanski et al., Tetrahedron Letters, 28:3401 (1987); Bannwarth, Helv. Chim. Acta., 71:1517 (1988); Crosstick and Vyle, Tetrahedron Letters, 30:4693

20 (1989); Agrawal, et al., Proc. Natl. Acad. Sci. USA, 87:1401-1405 (1990), the teachings of which are incorporated herein by reference. Other methods for synthesis or production also are possible. In a preferred embodiment the oligonucleotide is a

25 deoxyribonucleic acid (DNA), although ribonucleic acid (RNA) sequences may also be synthesized and applied.

The oligonucleotides useful in the invention preferably are designed to resist degradation by endogenous nucleolytic enzymes. In vivo degradation of

30 oligonucleotides produces oligonucleotide breakdown products of reduced length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to use

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oligonucleotides that are resistant to degradation in the body and which are able to reach the targeted cells. The present oligonucleotides can be rendered more resistant to degradation in vivo by substituting

5 one or more internal artificial internucleotide linkages for the native phosphodiester linkages, for example, by replacing phosphate with sulfur in the linkage. Examples of linkages that may be used include phosphorothioates, methylphosphonates, sulfone,

10 sulfate, ketyl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are illustrative, rather than limiting, since other internucleotide linkages are known in the art.

15 See, e.g., Cohen, Trends in Biotechnology (1990). The synthesis of oligonucleotides having one or more of these linkages substituted for the phosphodiester internucleotide linkages is well known in the art, including synthetic pathways for producing

20 oligonucleotides having mixed internucleotide linkages.

Oligonucleotides can be made resistant to extension by endogenous enzymes by "capping" or incorporating similar groups on the 5' or 3' terminal nucleotides. A reagent for capping is commercially available as

25 Amino-Link II<sup>TM</sup> from Applied BioSystems, Inc., Foster City, CA. Methods for capping are described, for example, by Shaw et al., Nucleic Acids Res., 19:747-750 (1991) and Agrawal, et al., Proc. Natl. Acad. Sci. USA, 88(17):7595-7599 (1991), the teachings of which are

30 hereby incorporated herein by reference.

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Methods of Application of the Oligonucleotides

In accordance with the invention, the inherent binding specificity of antisense oligonucleotides characteristic of base pairing is enhanced by limiting the availability of the antisense compound to its intended locus in vivo, permitting lower dosages to be used and minimizing systemic effects. Thus, oligonucleotides are applied locally to achieve the desired effect. The concentration of the oligonucleotides at the desired locus is much higher than if the oligonucleotides were administered systemically, and the therapeutic effect can be achieved using a significantly lower total amount. The local high concentration of oligonucleotides enhances penetration of the targeted cells and effectively blocks translation of the target nucleic acid sequences.

The oligonucleotides can be delivered to the locus by any means appropriate for localized administration of a drug. For example, a solution of the oligonucleotides can be injected directly to the site or can be delivered by infusion using an infusion pump. The oligonucleotides also can be incorporated into an implantable device which when placed at the desired site, permits the oligonucleotides to be released into the surrounding locus.

The oligonucleotides are most preferably administered via a hydrogel material. The hydrogel is noninflammatory and biodegradable. Many such materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body



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temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10 to about 80% by weight ethylene oxide and from about 20 to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic<sup>R</sup>.

In this embodiment, the hydrogel is cooled to a liquid state and the oligonucleotides are admixed into the liquid to a concentration of about 1 mg oligonucleotide per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, e.g., by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the oligonucleotides diffuse out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

The oligonucleotides can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be

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used to locally deliver the oligonucleotides. The oligonucleotides can be incorporated into the material as it is polymerized or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

As described in the following examples, the dose of oligonucleotides is dependent on the size of the oligonucleotides and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of oligonucleotide is somewhat dependent on the length and chemical composition of the oligonucleotide but is generally in the range of about 30 to 3000  $\mu\text{g}$  per square centimeter of tissue surface area. Based on calculations using the application of antisense myb in a hydrogel to blood vessel that has been injured by balloon angioplasty in a rat model, a dose of about 320  $\mu\text{g}$  oligonucleotide applied to one square centimeter of tissue was effective in suppressing expression of the c-myb gene product.

The oligonucleotides may be administered to the patient systemically for both therapeutic and prophylactic purposes. For example, antisense oligonucleotides specific for c-myb, NMMHC and/or PCNA may be administered to a patient who is at risk for restenosis due to angioplasty or other procedure. The oligonucleotides may be administered by any effective method, for example, parenterally (e.g., intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the oligonucleotides to access and circulate in the patient's bloodstream.

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Oligonucleotides administered systemically preferably are given in addition to locally administered oligonucleotides, but also have utility in the absense of local administration. A dosage in the range of from  
5 about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

#### Therapeutic Applications

The method of the present invention can be used to  
10 treat a variety of disorders which are linked to or based on expression of a protein by a gene. The method is particularly useful for treating vascular disorders, particularly vascular restenosis. The following non-limiting examples demonstrate use of antisense  
15 oligonucleotides to prevent or very significantly inhibit restenosis following vascular injury such as is induced by balloon angioplasty procedures. This has been accomplished by using antisense, delivered locally, to inhibit expression of genes encoding  
20 proteins determined to be involved in vascular restenosis, including c-myb, non-muscle myosin heavy chain (NMMHC) and proliferative cellular nuclear antigen (PNAC). However, the methods of the invention have many other uses.

25 Expression of specific genes in specific tissues may be suppressed by oligonucleotides having a nucleotide sequence complementary to the mRNA transcript of the target gene. Both c-myb and non-muscle myosin proteins appear to be critically involved  
30 in the initiation of proliferation of smooth muscle cells. The inhibition of the production of these proteins by antisense oligonucleotides offers a means for treating post-angioplasty restenosis and chronic processes such as atherosclerosis, hypertension,

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primary pulmonary hypertension, and proliferative glomerulonephritis, which involve proliferation of smooth muscle cells.

Illustrative of other conditions which may be  
5 treated with the present method are pulmonary disorders such as acute respiratory distress syndrome, idiopathic pulmonary fibrosis, emphysema, and primary pulmonary hypertension. These conditions may be treated, for example, by locally delivering appropriate antisense  
10 incorporated in an aerosol by inhaler. These disorders are induced by a complex overlapping series of pathologic events which take place in the alveolus (air side), the underlying basement membrane and smooth muscle cells, and the adjacent endothelial cell surface  
15 (blood side). It is thought that the alveolar macrophage recognizes specific antigens via the T cell receptor, become activated and elaborates a variety of substances such as PDGF which recruit white blood cells as well as stimulate fibroblasts. White cells release  
20 proteases which gradually overwhelm the existing antiproteases and damage alveolar pneumocytes; fibroblasts secrete extracellular matrix which induce fibrosis. Selected growth factors such as PDGF and the subsequent decrease in blood oxygen, which is secondary  
25 to damage to the alveolar membrane, induce smooth muscle growth. This constricts the microvascular blood vessels and further decreases blood flow to the lung. This further decreases the transport of oxygen into the blood. The molecular events outlined above also induce  
30 activation of the microvascular endothelial cell surface with the appearance of selectins and integrins as well as the appearance of tissue factor which initiates blood coagulation. These selectin and integrin surface receptors allow white blood cells to

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adhere to microvascular endothelial cells and release proteases as well as other molecules which damage these cells and allow fluid to accumulate within the alveolus. The above events also trigger microvascular  
5 thrombosis with closure of blood vessels. The end result of this process is to further impede oxygen exchange.

Antisense oligonucleotides, locally delivered to the alveolar/microvascular area, could be directed  
10 against the following targets to intervene in the pathology outlined above, since the cDNA sequences of all of the targets selected are known. Thus, antisense oligonucleotides specific for mRNA transcribed from the genes would inhibit production of the alveolar  
15 macrophage T cell receptor to prevent initiation of the above events; inhibit product of a protein to prevent activation of alveolar white cells, or inhibit production of elastase to prevent destruction of alveolar membrane; inhibit production of PDGF to  
20 prevent recruitment of white cells or resultant fibrosis; inhibit production of c-myc to suppress SMC proliferation; inhibit production of p-selectin or e-selectin or various integrins to prevent adhesion of blood white cells to pulmonary microvascular  
25 endothelial cells; or inhibit the production of tissue factor and PAI-1 to suppress microvascular thrombosis.

As additional examples, Tissue Factor (TF) is required for coagulation system activation. Local application of antisense targeting the mRNA or DNA of a  
30 segment of TF in the area of clot formation can prevent additional coagulation. This therapy can be employed as an adjunct to or as a substitute for systemic anticoagulant therapy or after fibrinolytic therapy, thereby avoiding systemic side effects.

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Plasminogen activator inhibitor (PAI-1) is known to reduce the local level of tissue plasminogen activator (TPA). The human cDNA sequence for PAI-1 is known. Local application of antisense targeting the mRNA or  
5 DNA of PAI-1 should permit a buildup of TPA in the targeted area. This may result in sufficient TPA production to naturally lyse the clot without systemic side effects.

A combination of antisense-TF and antisense-PAI-1  
10 may be utilized to maximize the efficacy of treatment of several disorders, including local post thrombolytic therapy and preventative post-angioplasty treatment.

Many other vascular diseases can be treated in a manner similar to that described above by identifying  
15 the target DNA or mRNA sequence. The treatment of diseases which could benefit using antisense therapy include, for example, myocardial infarction, peripheral muscular disease and peripheral angioplasty, thrombophlebitis, cerebro-vascular disease (e.g.,  
20 stroke, embolism), vasculitis (e.g., temporal arteritis) angina and Budd-Chiari Syndrome.

The present method can be used against a variety of targets in addition to those detailed above. For example, DNA or mRNA encoding the following proteins  
25 could be used as target sequences:

growth factors and receptors, including: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-alpha Receptor, PDGF-beta Receptor, k-FGF(hst), int-2, bFGF, bFGF Receptor, aFGF, aFGF Receptor, TGF-beta family (TGF beta 1,2,3, and  
30 others), TGF-beta Receptors (type I and type II), EGF, EGF Receptor (erbB-2, her, neu), erbB3, Amphiregulin, Amphiregulin receptor, Heparin binding growth factor (HBGF), HBGF Receptor, Thrombin, Thrombin Receptor, Serum-derived growth factor (SDGF), SDGF Receptor,

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- Interleukins (IL-1,2,3,4,5,6,7,8,9,10,11), IL Receptors, Steroid Receptor, CSF, CSF Receptor, IGF-1, IGF-1 Receptor, IGF-2, IGF-2 Receptor, Insulin Receptor, ros, erba (thyroid hormone receptor), HGF,
- 5 HGF receptor, wnt, wnt receptor, c-kit, stl;  
cell cycle specific proteins, including: G1, c-myc, N-myc, ski, c-fos, c-jun, jun B, jun D, AP1, c-myb, cdc2, G1 cyclins, PCNA, cyclin E, p34, Rb, rel, CLN1 and equivalent, CLN2 and equivalent, c-ras, p120, cyclin
- 10 D1, Prad1 gene, EGR-1, S, DNA polymerase alpha, DNA polymerase delta, DNA polymerase, other subunits, RF-C, Helicase, DNA primase, RF-A, RP-C, DNA topoisomerase I, II, CAF-1, p55 antigen, Ki67 antigen, B23, C23, Dihydrofolate reductase, Thymidylate synthetase,
- 15 Ribonucleotide reductase (M1 and M2 subunits),  
Thymidine kinase, 4F2 antigen, 5E9 antigen, 2-5A oligosynthetase, G2/M, cyclin A, cyclin B, wee 1, cdc 25, nim1, PTP 1B, PP1, PP2A, p105 Antigen, Calmodulin, Calmodulin kinase I, II, Actins: beta, gamma, alpha,
- 20 Tubulin, Microtubule-associated antigen, p21, Myosin heavy chain: alpha, beta, A, B, embryonic, etc., Myosin non-regulatory light chain, Myosin essential light chain, Myosin light chain kinase, Myosin phosphatase, CAMP kinase, CAMP phosphatase, Myosin heavy chain
- 25 kinase, 62 kDa Ca-binding protein, Calpain II, Dynein, Kinesin, INCENP proteins;  
signal transduction factors, including: src, MAP4, MAP Kinase, GMRF, GAP, NF-1, NF-kappa B, PI-3K, PLC-gamma, SRF, yes, fps (fes), abl, met, mos, raf (mil), Ha-ras,
- 30 Ki-ras, N-ras, crk;  
adhesion proteins, including: ICAM-1, VCAM, LCAM, Integrin Family, Tensin, FAK, Thrombomodulin; and calcium-related factors, including: Na/Ca exchanger, CA ATPase pump, L channel, T channel, and other Ca
- 35 pumps and channels.

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The following Examples are included by way of illustration, and are not intended to limit the scope of the invention.

#### EXAMPLES

5

##### Materials and Methods

SV40LT-SMC (rat smooth muscle cells, gift of Dr. C. Reilly, Merck, Sharp and Dohme, West Point, PA) were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Bethesda, MD). BC3H1 mouse smooth muscle cells (ATCC CRL 1443, obtained from the American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS). The cells were cultured at 37°C in a humidified 5% CO atmosphere.

Primary aortic smooth muscle cells (SMC) were isolated by the explant technique from Sprague-Dawley rats (average weight 350 g) and FVB mice (average weight 50 g). Ross, J. Cell Biol., 50:172-186 (1971). The cultures exhibited typical morphological characteristics of vascular SMC (spindle shape and hill-and-valley pattern). Identification of vascular SMC was confirmed by Northern analysis demonstrating the presence of the smooth muscle alpha actin isoform. Primary aortic SMC were used in the second passage.

Antisense and sense 18-mer phosphorothiolate oligonucleotides were synthesized on an ABI DNA synthesizer. Oligonucleotides were deprotected on the machine, dried down, resuspended in "TE" (10 mM Tris, pH 7.5, 1mM EDTA, pH8.0) and quantified by spectrophotometry and gel electrophoresis. The following sequences were employed:



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Antisense c-myb oligonucleotide:

Sequence ID NO. 1

5 GTGTCGGGGTCTCCGGGC

Antisense NMMHC oligonucleotide:

Sequence ID No. 2

10 CATGTCCTCCACCTTGA

Antisense thrombomodulin ("TM") oligonucleotide:

15 Sequence ID No. 3

ACCCAGAAAGAAAATCCCAAG

and, Antisense human c-myb oligonucleotide, which has 2  
20 mismatches compared with mouse c-myb:

Sequence ID No. 4

25 GTGCCGGGGTCTTCGGGC.

The Sequence ID No. 1 is complementary to nucleotides  
4-22 of mouse c-myb (Bender et al., (1986) Natl. Acad.  
Sci. USA, 83:3204-3208); Sequence ID No. 2 is  
complementary to nucleotides 232-250 of human NMMHC-A  
30 (Simons et al., (1991) Cir. Res., 69:530-539); and  
Sequence ID No. 3 is complementary to nucleotides 4-25  
of mouse TM (Dittman and Majerus, (1989) Nucl. Acid  
Res., 17:802). Sequence ID No. 4 is complementary to a  
human c-myb sequence and has 2 base mismatches compared

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with murine c-myb. The NMMHC sequence was chosen in a region with the closest degree of homology between known nonmuscle myosin sequences. The sequence has 1 nucleotide difference with a human NMMHC-B (Simons et al., ibid.) and 2 nucleotide differences with chicken NMMHC-A and NMMHC-B. The corresponding sense sequences were used as controls.

Example 1: Inhibition of c-myb and NMMHC-A using antisense oligonucleotides in vitro.

#### Growth Assay

Both cell lines as well as early passage primary aortic SMC were seeded at a density of 25,000 cells per well in cluster 6 well plates (Costar, Cambridge, MA) in 10% FBS-DMEM (20% FBS-DMEM for BC3H1 cells). The following day, the cells were washed twice with phosphate-buffered saline (PBS), the media was replaced with 0.5% FBS-DMEM growth arrest media, and the cells were kept in growth-arrest media for 96 hours. The media then was changed to 10% or 20% FBS-DMEM, and synthetic c-myb and NMMHC antisense and sense oligonucleotides were added. The cells were permitted to grow for 72 hours, trypsinized and counted on a Coulter Counter.

Alternatively, the two cell lines and the SMC cells were allowed to proliferate in 10% or 20% FBS-DMEM, oligonucleotides were added, and cell counts were obtained after 5-8 days as described above. Each experiment was carried out in triplicate and repeated at least two additional times. Data is expressed as mean  $\pm$  standard deviation.

- 25 -

The results (shown in Figure 1) showed that, in vitro, antisense oligonucleotides to both c-myb (Seq. ID No. 1) and NMMHC (Seq. ID No. 2) caused substantial suppression of cellular proliferation while the sense oligonucleotides had no effect and were similar to the results obtained using just Tris-EDTA buffer.

The oligonucleotides utilized were derived from the nucleotide sequences of human/chicken NMMHC or mouse c-myb cDNAs. The importance of the specificity of the antisense oligonucleotides was shown by the complete loss of antiproliferative action when two bases of the 18 base c-myb antisense sequence were randomly altered (Seq. ID No. 4). The results for this test were as follows: antisense c-myb: 475,600 cells  $\pm$  25,000 cells; mismatch antisense c-myb: 958,800 cells  $\pm$  12,000 cells; sense c-myb: 935,200 cells  $\pm$  22,000 cells. Thus, the mismatch antisense c-myb (seq. ID No. 4) failed to inhibit proliferation of SMC cells significantly. The antisense and sense phosphorothiolate thrombomodulin (TM) oligonucleotides had no apparent effect on SMC growth (antisense TM: 364,580 cells  $\pm$  19,000 cells vs sense TM: 376,290 cells  $\pm$  11,000 cells).

The inhibitory action of antisense phosphorothiolate oligonucleotides directed against NMMHC as compared to c-myb was more clearly concentration-dependent (antisense NMMHC: 32% vs 65% suppression at 2 $\mu$ M and 25 $\mu$ M, respectively; antisense c-myb: 33% vs 50% suppression at 2 $\mu$ M and 25 $\mu$ M respectively). Previous estimates of the relative abundance of these two messages indicated that c-myb mRNA occurs at extremely low concentrations in exponentially growing SMC (less than 0.01% of poly A+ RNA), whereas NMMHC mRNA is present at significantly

- 26 -

higher levels. The observed concentration dependence of the two antisense oligonucleotides with regard to growth inhibition was consistent with the relative abundance of the two mRNAs.

5       The antiproliferative effects of the antisense and sense phosphorothiolate oligonucleotides were also evaluated with the BC3H1 cell line as well as with primary rat and mouse aortic SMC. The data obtained showed that growth of the three cell types is greatly  
10 suppressed with phosphorothiolate antisense but not sense NMMHC or c-myb oligonucleotides (Figure 2). Antisense c-myb oligonucleotides exhibited a greater antiproliferative effect on mouse aortic SMCs and mouse BC3H1 cells as compared to rat aortic SMC and rat  
15 SV40LT-SMC (Figure 2B). The difference in growth inhibition is most likely attributable to the greater extent of antisense nucleotide mismatch between rat and mouse c-myb sequences within the chosen area.

      The minimal time required for exposure of  
20 SV40LT-SMC to antisense NMMHC or c-myb phosphorothiolate oligonucleotides to achieve maximal growth inhibition was determined. In the studies cited above, cells were continuously exposed to oligonucleotides from the time of shift from growth  
25 arrest media by addition to 10%FBS-DMEM to the measurement of antiproliferative effect by cell count at 72 hr. In the experiments cited below, SV40LT-SMC were treated with antisense oligonucleotides for stated periods after the shift from growth arrest, washed  
30 twice with PBS, placed in fresh oligonucleotide free 10% FBS-DMEM, and assessed for growth inhibitory effect by cell count at 72 hr. The results, shown in Figure 3A showed that the addition of 25 $\mu$ M antisense NMMHC or c-myb oligonucleotides for as little as 1 hr, generated

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- a significant antiproliferative effect. After 2 hr of treatment with antisense NMMHC oligonucleotides, the extent of growth inhibition is equivalent to that obtained by continuous exposure for 72 hr (65% suppression of cell growth). The admixture of antisense c-myb oligonucleotides for 4 hr produced an antiproliferative effect which is identical to that observed with continuous exposure for 72 hr (50% suppression of cell growth).
- 10 An experiment was performed to determine whether the growth inhibitory effects of antisense phosphorothiolate oligonucleotides are readily reversible. To this end, SV40LT-SMC were exposed for 4 hr after release from growth arrest to 25  $\mu$ M
- 15 antisense or sense NMMHC or c-myb oligonucleotides. The cells were subsequently washed twice with PBS, placed in fresh oligonucleotide free 10% FBS-DMEM media, and cell counts were determined at day 3 and day 5. The data revealed that proliferation of SV40LT-SMC treated with antisense NMMHC or c-myb, as compared
- 20 to the corresponding sense oligonucleotides, demonstrated a significant initial suppression of growth at day 3 of about 65% and 50%, respectively. However, the doubling times, between day 3 and day 5 of the SV40LT-SMC treated with antisense NMMHC or c-myb, as compared to the corresponding sense
- 25 oligonucleotides, were identical at 22 hrs.
- Treatment of SV40LT-SMC with 25  $\mu$ M unmodified antisense NMMHC or c-myb oligonucleotides for 16 hr
- 30 after release from growth arrest, produced no discernible antiproliferative effect at 72 hr. In contrast, the continuous exposure for 40 hr to the two oligonucleotides, at identical levels, resulted in the same growth inhibitory effect observed with the
- 35 phosphorothiolate derivatives (Figure 3B).

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The importance of growth arrest to the antiproliferative potency of antisense NMMHC and c-myb phosphorothiolate oligonucleotides also was evaluated. SV40LT-SMC were allowed to grow exponentially while  
5 continuously exposed to 10  $\mu$ M antisense NMMHC or c-myb oligonucleotides and cell counts were determined at 72 hr and 120 hr. The treatment of SMC with antisense NMMHC oligonucleotides produced no growth inhibitory effect at either time point, whereas exposure to  
10 antisense c-myb oligonucleotides generated a 19% suppression of proliferation at 72 hr and a 40% suppression of proliferation at 120 hr.

#### RNA Analysis

15 Total cellular RNA was determined from SV40LT-SMC cells in culture 24 hours after growth induction with 10% FBS in DMEM using the method of Chomzynski and Sacchi, J. Cell. Physiol., 142:342 (1990). The RNA was quantified by spectrophotometry and a total of 10  $\mu$ g  
20 was applied to nitrocellulose using a dot blot apparatus. The blot was then hybridized with a random primed c-myb, NMMHC, large T-antigen, GAPDH and TM probes in 10% dextran sulfate and 40% formamide for 16 hours at 42°C. Northern blots and RNA dot blots were  
25 washed in SSC solution with final washes performed at 50°C and 0.5 x SSC for c-myb, 55°C and 0.2 x SSC for NMMHC, 55°C and 0.2 x SSC for large T antigen, 50°C and 0.2 x SSC for GAPDH and 50°C and 0.2 x SSC for TM. The Northern blots were subjected to autoradiography. The  
30 RNA blots were quantified by normalizing c-myb or GAPDH message counts to large T-antigen counts using a Betascope 603 analyzer (Betagen, Waltham, MA). The numbers represent total counts for each dot. The entire experiment was repeated twice. Results are for

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cells treated with sense c-myb oligonucleotide (25  $\mu$ M); antisense c-myb oligonucleotide (25  $\mu$ M); and heparin (100  $\mu$ g/ml). The cells were allowed to reach confluence and become quiescent for two days.

- 5        Cells exposed to antisense c-myb oligonucleotide had a markedly decreased amount of c-myb message present as assessed by RNA dot blot hybridization with a radiolabeled c-myb probe. The results are shown in Figure 4. Figure 4 shows individual dot counts, adjusted for the quantity of RNA in each sample, for 10        synchronized proliferating SV-SMCs treated with sense c-myb (S Myb) and antisense c-myb (AS Myb), heparin-arrested (heparin), and growth-arrested (GA) cells. Cell growth was arrested with antisense c-myb to about 15        the same degree as with heparin had similar amounts of c-myb message present. Similarly, antisense NMMHC oligonucleotide led to a marked attenuation of the NMMHC message detected on a Northern blot.

- 20        The amount of c-myb protein in the cells treated with antisense c-myb was markedly reduced, as was the non-muscle myosin protein in cells treated with antisense NMMHC-B, as assessed by indirect immunofluorescence.

25        Immunofluorescence Microscopic Examination of SMC for c-myb and nonmuscle myosin

- SV40LT-SMC were fixed with 2% formaldehyde/PBS at room temperature for 15 minutes, permeated with 2% Triton X-100/PBS, washed 3 times with 1% BSA/PBS and 30        exposed for 2-4 hr to anti-myb or anti-NMMHC antisera diluted 1:250 or 1:1000 in 1% BSA/PBS. The anti-myb antisera was obtained from Cambridge Research Laboratories (Wilmington, DE) and was generated by immunizing rabbits with the synthetic peptide His-Thr-

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Cys-Ser-Tyr-Pro-Gly-Trp-His-Ser-Thr-Ser-Ile-Val  
corresponding to mouse c-myb amino acid residues 332-  
345. The anti-nonmuscle myosin antiserum was kindly  
provided by RS Adelstein and JS Sellers (LMC, NIH,  
5 Bethesda, MD) and was generated by immunizing rabbits  
with purified human platelet myosin. This antiserum is  
monospecific as judged by Western blot analysis. The  
cells were washed three times with 1% BSA/PBS to remove  
excess primary antibody followed by incubation for 2 hr  
10 with second antibodies diluted 1:100 in 1% BSA/PBS  
(rhodamine-conjugated goat anti-rabbit IgG and FITC-  
conjugated sheet anti-rabbit purchased from Organon  
Teknika, Durham, NC). After washing cells three times  
with 1% BSA/PBS, the samples were examined with a Nikon  
15 Optiphot fluorescence photomicroscope.

The reduction in the concentrations of NMMHC and  
c-myb mRNAs induced by antisense oligonucleotides  
should lead to a decrease in the levels of the specific  
proteins. To show this effect, SV40LT-SMC cells were  
20 plated at low density ( $10,000/\text{cm}^2$ ) on a 2 well glass  
slides (Nunc, Inc., Naperville, IL), growth-arrested  
for 96 hr in 0.5% FBS-DMEM and then shifted to 10% FBS-  
DMEM to which were added 25  $\mu\text{M}$  antisense or sense NMMHC  
or c-myb phosphorothiolate oligonucleotides. After  
25 24 hr (c-myb) or 72 hr (NMMHC), SMC were examined by  
indirect immunofluorescence microscopy utilizing  
specific antisera against NMMHC or c-myb. It was  
readily apparent that the concentrations of NMMHC or  
c-myb proteins in most cells treated with antisense  
30 oligonucleotides were dramatically reduced as compared  
to those exposed to sense oligonucleotides. However,  
it was also clear that occasional cells in each panel  
displayed substantial amounts of protein. This  
observation was probably secondary to the premature



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escape of this subpopulation from growth arrest and the subsequent augmentation in their levels of target mRNAs. The specificity of the immunofluorescence technique was documented by carrying out the above  
5 procedure with untreated SMC in the absence of primary antibody or in the presence of a large excess of purified antigen which showed minimal background signal.

10 Example 2: Release of oligonucleotides from polymeric matrices.

Release of Oligonucleotides from  
Pluronic<sup>TM</sup> Gel Matrix

15 Matrices made from a poly (ethylenoxide-propyleneoxide) polymer containing c-myb and NMMHC antisense oligonucleotides (described in Materials and Methods) were prepared in order to test the rate of release of the oligonucleotides from the  
20 matrices. The test samples were prepared by weighing 1.25 g of UV sterilized Pluronic<sup>TM</sup> 127 powder (BASF Corp., Parsippany, N.J.) in scintillation vials and adding 3.25 ml of sterile water. Solubilization was achieved by cooling on ice while shaking. To these  
25 solutions were added 500  $\mu$ l of a sterile water solution containing the oligonucleotides (5.041 mg/500 $\mu$ l). The final gels contained 25% (w/w) of the polymer and 1mg/g oligonucleotides.

The release kinetics of the gels containing  
30 oligonucleotides were determined by placing the gels in PBS and measuring the absorption (OD) over time. The results for four test gels, shown in Figure 5, indicate that oligonucleotides are released from the gels in less than one hour.

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Release of oligonucleotides from EVAc matrices

The release of oligonucleotides from ethylene vinyl acetate (EVAc) matrices was demonstrated.

5        Matrices were constructed and release was determined as described by Murray et al. (1983), In Vitro., 19:743-748. Ethylene-vinyl acetate (EVAc) copolymer (ELVAX 40P, DuPont Chemicals, Wilmington, DE) was dissolved in dichloromethane to form a 10% weight  
10 by volume solution. Bovine serum albumin and the oligonucleotide were dissolved together at a ratio of 1000 - 2000:1 in deionized H<sub>2</sub>O, frozen with liquid N and then lyophilized to form a dry powder. The powder was pulverized to form a homogeneous distribution of  
15 particles less than 400 microns in diameter. A known quantity of the powder was combined with 4-10 ml of the 10% (w/v) EVAc copolymer solution in a 22 ml glass scintillation vial. The vial was vortexed for 10  
20 seconds to form a homogeneous suspension of the drug particles in the polymer solution. This suspension was poured onto a glass mold which had been precooled on a slab of dry ice. After the mixture froze it was left in place for 10 minutes and then removed from the mold and placed into a -20°C freezer for 2 days on a wire  
25 screen. The slab was dried for an additional 2 days at 23°C under a 600 millitorr vacuum to remove residual dichloromethane. After the drying was complete 5 mm X 0.8 mm circular slabs are excised with a #3 cork borer.

30        The results, shown in Figure 6, indicate that about 34% of the oligonucleotide was released within the first 48 hours.

Example 3: In vivo application of oligonucleotides to inhibit c-myc and NMMHC in rats.

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## I. Animal Model.

Balloon stripping of the rat carotid artery is used as a model of restenosis in vivo. Rats were  
5 anesthetized with Nembutal (50 mg/kg). A left carotid dissection was carried out and a 2F Fogarty catheter was introduced through the arteriotomy incision in the internal carotid artery. The catheter was advanced to the aortic arch, the balloon was inflated and the  
10 catheter withdrawn back to the arteriotomy site. This was repeated two more times. Subsequently, the balloon being withdrawn, the internal carotid was tied off, hemostasis achieved, and the wound closed.

## 15 II. Oligonucleotide Delivery.

The oligonucleotides were applied with a hydrogel and with an implantable ethylene vinyl acetate (EVAc) matrix. A polyethylene oxide-polypropylene oxide polymer (Pluronic<sup>TM</sup> 127, BASF, Parsippany, NJ) was used  
20 as a hydrogel. The Pluronic<sup>TM</sup> gel matrices were prepared as described in Example 2. Briefly, sterile solutions of Pluronic<sup>TM</sup> 127 were prepared by weighing 1.25 g of UV sterilized Pluronic powder into a scintillation vial and adding 3.25 ml of sterile water.  
25 Solubilization was achieved by cooling on ice while shaking, forming a solution containing 27.7% by weight of the polymer. To these solutions were added 500  $\mu$ L of a sterile water solution of the antisense c-myb (See Example 1) oligonucleotides (5.041 mg/500  $\mu$ L). The  
30 final gels were 25% w/w of Pluronic<sup>TM</sup> polymer and 1mg/g oligonucleotide. Drug-free 25% (w/w) gels were prepared as controls. The EVAc matrices were prepared as described in Example 2, and contained 40  $\mu$ g of oligonucleotide.

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Immediately after balloon injury, 200  $\mu$ l of Pluronic/oligonucleotide solution (which contained 200  $\mu$ g of the oligonucleotide) was applied to the adventitial surface of the artery and gelling was allowed to occur. The antisense/EVAc matrix (which contained 40  $\mu$ g of the oligonucleotide) and drug-free gels were applied in the same manner.

### III. Quantification of Effect.

After 14 days, the animals were sacrificed and the carotid arteries were perfused under pressure (120 mmHg) with Ringer's Lactate. Both carotid arteries were excised and fixed in 3% formalin. Thin sections were then prepared for light microscopy in a standard manner. The slide was visualized and digitized using a dedicated computer system and by a hand held plenumeter and the area of neointimal proliferation calculated (in sq mm).

The results are shown in Figure 7. In control animals which received no treatment, or which were treated with the drug-free gel, there was extensive restenosis, characterized by symmetric neointimal formation along the entire length of the injured artery, narrowing the lumen by about 60%, resulting in an intima/media ratio of 1.4.

In animals treated with antisense c-myb oligonucleotides, there was minimal restenosis, minimal proliferative rim (less than 10% of the lumen) that was limited to the portion of the artery in direct contact with oligonucleotide, with an intima/media ratio of 0.09. As shown in Figure 7, this effect was most pronounced for animals treated with the antisense/Pluronic<sup>R</sup>. The intima/media ratio obtained using EVAc/antisense was about 0.45. However, the EVAc

- 35 -

matrix contained 40  $\mu$ g of oligonucleotide, compared with 200  $\mu$ g of oligonucleotide administered in the Pluronic gel, which may account for some of the difference.

5        Figure 8 shows the results of extension of this experiment, in which 28 rats were treated as described above. Seven rats in each treatment group were subjected to balloon angioplasty, and the arterial walls treated as follows: with a drug free hydrogel  
10    (Pluronic<sup>TM</sup> 127 as described above), a hydrogel containing sense c-myb, a hydrogel containing antisense c-myb, and no treatment at all. As shown in Figure 8, similar high levels of neointimal proliferation occurred in all animals except those treated with  
15    antisense c-myb, where the levels of proliferation were dramatically lower.

Example 4: Inhibition of PCNA using antisense oligonucleotides.

20

Using the same methodology as in Example 1, antisense for PCNA having the sequence:

Sequence ID No. 5:

25

GAT CAG GCG TGC CTC AAA,

was applied to SV-SMC cells in culture. Sense PCNA was used as a negative control; NMMHC-B was used as a  
30    positive (inhibitory) control.

There was no suppression of smooth muscle cell proliferation in the negative control; there was 52% suppression using antisense NMMHC-B and 58% suppression with antisense PCNA.

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Example 5: In vivo application of antisense oligonucleotides to inhibit smooth muscle cell proliferation in rabbits.

5 New Zealand white rabbits (1-1.5 Kg) were anesthetized with a mixture of ketamine and zylazine and carotid dissection was performed as described in Example 3. A 5F Swan-Ganz catheter was inserted and  
10 positioned in the descending aorta with fluoroscopic guidance. The Swan-Ganz catheter was exchanged over the wire for an angioplasty catheter with a 3.0 mm balloon. The common iliac artery was angioplastied 3 times at 100 PSI for 90 seconds each time. A  
15 Wolinsky catheter was introduced and loaded with oligonucleotide solution in a total volume of 5cc normal saline. Saline was injected as a control in a counterlateral iliac artery. The oligonucleotides were a mixture of antisense mouse c-myb and human NMMHC  
20 (200 $\mu$ M of each), described above. The mixture was injected under 5 atmospheres of pressure over 60 seconds. Two rabbits were treated with antisense oligonucleotide.

25 The animals were sacrificed 4 weeks later and the arteries were processed as described in Example 3 for rat arteries.

The results, shown in Figure 9 indicated a 50% reduction in neointimal proliferation in rabbit arteries treated with antisense compared to saline  
30 alone.

Example 6. Inhibition of proliferation of baboon smooth muscle cells using antisense oligonucleotides.

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Using the same methodology as in Example 1, primary baboon smooth muscle cells (gift from Dr. Hawker, Emory University) were treated with antisense human myb (Seq. ID No. 4) and human NMMHC (Seq. ID No. 2). The cells  
5 were allowed to grow for 72 hours after treatment with the oligonucleotides, then counted as described in Example 1. The results show that hNMMHC caused 65.5% growth suppression and c-myb caused 59.77% growth suppression in the baboon cells.

10

#### Equivalents

One skilled in the art will recognize several equivalents, modifications, variations of the present  
15 method from the foregoing detailed description. Such equivalents, modifications and variations are intended be encompassed by the following claims.

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## SEQUENCE LISTING

## 5 (1) GENERAL INFORMATION:

- (i) APPLICANT:  
ROSENBERG, ROBERT D.  
SIMONS, MICHAEL  
EDELMAN, ELAZER  
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DEKEYSER, JEAN-LUC
- (ii) TITLE OF INVENTION: LOCALIZED OLIGONUCLEOTIDE THERAPY
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:  
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(D) STATE: MASSACHUSETTS  
(E) COUNTRY: U.S.A.  
(F) ZIP: 02109
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: US  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 07/792,146  
(B) FILING DATE: 8 NOVEMBER 1991
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 07/723,454  
(B) FILING DATE: 28 JUNE 1991
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- (ix) TELECOMMUNICATION INFORMATION:  
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(2) INFORMATION FOR SEQ ID NO:1:

```

5      (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 bases
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

10     (ii) MOLECULE TYPE: DNA (synthetic)

        (iii) HYPOTHETICAL: NO

15     (iv) ANTI-SENSE: YES

        (ix) FEATURE:
        (A) NAME/KEY: Misc. Feature
        (B) LOCATION: 1 -18
20     (D) OTHER INFORMATION: /function= Antisense Sequence
        to mouse c-myb
        /standard_name= "Antisense mouse c-myb"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

25     GTG TCG GGG TCT CCG GGC

```

**(2) INFORMATION FOR SEQ ID NO:2:**

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18  
(B) TYPE: nucleic acid  
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

40 (iv) ANTI-SENSE: YES

(ix) FEATURE:  
(A) NAME/KEY: Misc. Feature  
(B) LOCATION: 1 - 18  
45 (D) OTHER INFORMATION: /function= Antisense Sequence  
to human NMMHC  
/standard name= "Antisense human NMMHC"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAT GTC CTC CAC CTT GGA

5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

10

- (A) LENGTH: 18 bases
- (B) TYPE: nucleic acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

15

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

20

(ix) FEATURE:

- (A) NAME/KEY: Misc. Feature
- (B) LOCATION: 1 - 21
- (D) OTHER INFORMATION: /function= Antisense Sequence  
to mouse thrombomodulin  
/standard\_name= "Antisense mouse thrombomodulin"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACC CAG AAA GAA AAT CCC AAG

30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35

- (A) LENGTH: 18 bases
- (B) TYPE: nucleic acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

40

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

45

(ix) FEATURE:

- (A) NAME/KEY: Misc. Feature
- (B) LOCATION: 1 - 18
- (D) OTHER INFORMATION: /function= Antisense Sequence  
to human c-myb.  
/standard\_name= "Antisense human c-myb"

50

- 41 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTG CCG GGG TCT TCG GGC

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 18 bases  
(B) TYPE: nucleic acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

15 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:

- 20 (A) NAME/KEY: Misc. Feature  
(B) LOCATION: 1 - 18  
(D) OTHER INFORMATION: /function= Antisense Sequence  
to PCNA  
/standard\_name= "Antisense PCNA"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAT CAG GCG TGC CTC AAA

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CLAIMS

1. A method of inhibiting translation or transcription of a target nucleic acid sequence  
5 preferentially at a locus in vivo, the method comprising:  
directly applying to a tissue at said locus within the body of a mammal an oligonucleotide, complementary to the target sequence, in an amount  
10 sufficient to penetrate cells of the tissue, to hybridize with said target nucleic acid, and to inhibit intracellular translation or transcription of said target sequence.
- 15 2. The method of claim 1 wherein the oligonucleotide is in a physiologically compatible solution and wherein it is applied by injection.
3. The method of claim 1 wherein the solution is  
20 applied to the tissue using an infusion pump, stent, or catheter.
4. The method of claim 1 wherein the tissue comprises smooth muscle tissue.  
25
5. The method of claim 1 wherein the oligonucleotide comprises an antisense sequence complementary to the sequence of a gene selected from the group consisting of c-myb, NMMHC and PCNA.  
30
6. The method of claim 1 wherein said oligonucleotide sequence comprises about 14 to 38 nucleotide bases.

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7. The method of claim 1 wherein the oligonucleotide is treated to render it resistant to degradation or extension by intracellular enzymes.

5       8. The method of claim 7 wherein the treatment comprises substituting at least one backbone phosphodiester linkage of the oligonucleotide with a linkage selected from the group consisting of phosphorothioate, methylphosphonate, sulfone, sulfate,  
10 ketyl, phosphorodithioate, various phosphoramidate, phosphate ester, bridged phosphorothioate and bridged phosphoramidate linkages.

9. The method of claim 7 wherein the treatment  
15 comprises capping a 3'-nucleotide with a structure resistant to addition of nucleotides.

10. The method of claim 1 wherein the oligonucleotide is delivered to the tissue in a  
20 concentration of between approximately 30 and 3000  $\mu$ g oligonucleotide per square centimeter of tissue surface area.

11. The method of claim 1 wherein the target  
25 nucleic acid sequence comprises an mRNA.

12. The method of claim 11 wherein the mRNA encodes a protein selected from the group consisting of c-myc protein, non-muscle myosin heavy chain and PCNA.

30

13. The method of claim 11 wherein said oligonucleotide sequence inhibits translation of the mRNA.

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14. A method of inhibiting translation or transcription of a target nucleic acid sequence preferentially at a locus of tissue in vivo, the method comprising:

5 directly depositing onto a surface of the tissue at said locus within the body of a mammal an oligonucleotide complementary to the target sequence; and

10 permitting the oligonucleotide to penetrate into cells of the tissue in an amount sufficient to combine with said target nucleic acid and to inhibit intracellular translation or transcription thereof.

15 15. The method of claim 14 wherein the oligonucleotide is delivered to the locus by injection, catheter, stent, or infusion.

20 16. The method of claim 14 wherein the oligonucleotide is incorporated into a carrier.

17. The method of claim 16 wherein the carrier comprises an implantable matrix.

25 18. The method of claim 16 wherein the carrier comprises a hydrogel.

30 19. The method of claim 18 wherein the hydrogel comprises a material which is liquid at a temperature below 37°C.

20. The method of claim 19 wherein the hydrogel material comprises a polyoxyethylene oxide-polypropylene oxide copolymer.

- 45 -

21. The method of claim 20 wherein the polymer comprises from about 10 to about 80% by weight polyethylene oxide and from about 20 to about 90% polyethylene oxide.

5

22. The method of claim 21 wherein the polymer comprises about 70% by weight polyethylene oxide and about 30% by weight polypropylene oxide.

10

23. The method of claim 1 wherein the oligonucleotide comprises about 14 to 38 nucleotide bases.

24. The method of claim 14 wherein the target  
15 nucleic acid comprises an mRNA sequence transcribed from a gene selected from the group consisting of c-myb, NMMHC and PCNA.

25 The method of claim 14 wherein the  
20 oligonucleotide is deposited extravascularly.

26. The method of claim 14 wherein said oligonucleotide is deposited onto or beneath an adventitial surface of the vascular system.

25

27. The method of claim 14 wherein said oligonucleotide inhibits translation of the target sequence.

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28. A method of inhibiting smooth muscle cell (SMC) proliferation in vivo in a target area of the vascular system comprising:

directly applying to tissue in the target area  
5 antisense oligonucleotide, complementary to a nucleic acid expressed in SMC's necessary to initiate or support proliferation thereof, at a concentration sufficient to induce selective antisense-nucleic acid combination within the SMCs for a time sufficient to  
10 inhibit proliferation.

29. The method of claim 28 wherein the nucleic acid comprises mRNA encoding a protein selected from the group consisting of c-myb protein, non-muscle  
15 myosin and PCNA.

30. An implant for local sustained delivery of antisense oligonucleotides comprising a carrier containing antisense oligonucleotides.  
20

31. The implant of claim 30 wherein the carrier comprises a polymer.

32. The implant of claim 31 wherein the polymer  
25 comprises a hydrogel material which is liquid at temperatures below about 37°C and gels at body temperature.

33. The implant of claim 32 wherein the hydrogel  
30 comprises a polyethylene oxide - polypropylene oxide copolymer.



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34. The device of claim 30 wherein the oligonucleotide is present at a concentration in said polymer sufficient to release into a surface on which it is deposited from about 30 to about 3000  $\mu\text{g}$  oligonucleotide per square centimeter of tissue surface area.

35. Oligonucleotides adapted for cell penetration and resistant to intracellular degradation comprising nucleotide bases complementary to mRNA encoding a protein selected from the group consisting of non-muscle myosin and PCNA.

36. Oligonucleotides adapted for cell penetration and resistant to intracellular degradation comprising nucleotide bases complementary to an intracellular nucleic acid which expresses a protein necessary for initiation or support of smooth muscle cell proliferation.

37. The oligonucleotides of claim 36 wherein the protein comprises c-myb protein, non-muscle myosin, or PCNA.

38. An implantable device for local sustained delivery of antisense oligonucleotides comprising a biocompatible carrier in combination with an oligonucleotide complementary to a target nucleotide sequence in a tissue.

- 48 -

39. The device of claim 38 wherein the carrier comprises a polymer selected from the group consisting of polyanhydrides, polyorthoesters, polylactic acid, polyglycolic acid, poly (lactic acid-glycolic acid) copolymers, collagen, dextran, protein polymers, ethylenevinyl acetate, polyvinyl acetate, polyvinyl alcohol, polymers of propylene glycol and ethylene oxide, and derivatives thereof.

40. The device of Claim 38 wherein the target nucleotide sequence encodes a molecule which can induce restenosis.

41. The device of claim 38 wherein the target nucleotide sequence encodes a molecule which can induce thrombosis.

42. The device of claim 38 wherein the target nucleotide sequence encodes a molecule involved in development of a pulmonary disorder.

43. The device of claim 38 wherein the target nucleotide sequence encodes a molecule involved in development of a cardiovascular disorder.

44. Use of an oligonucleotide which is complementary to a target nucleotide sequence in smooth muscle tissue for the manufacture of a medicament for inhibiting translation or transcription of the target nucleotide sequence in the smooth muscle tissue.

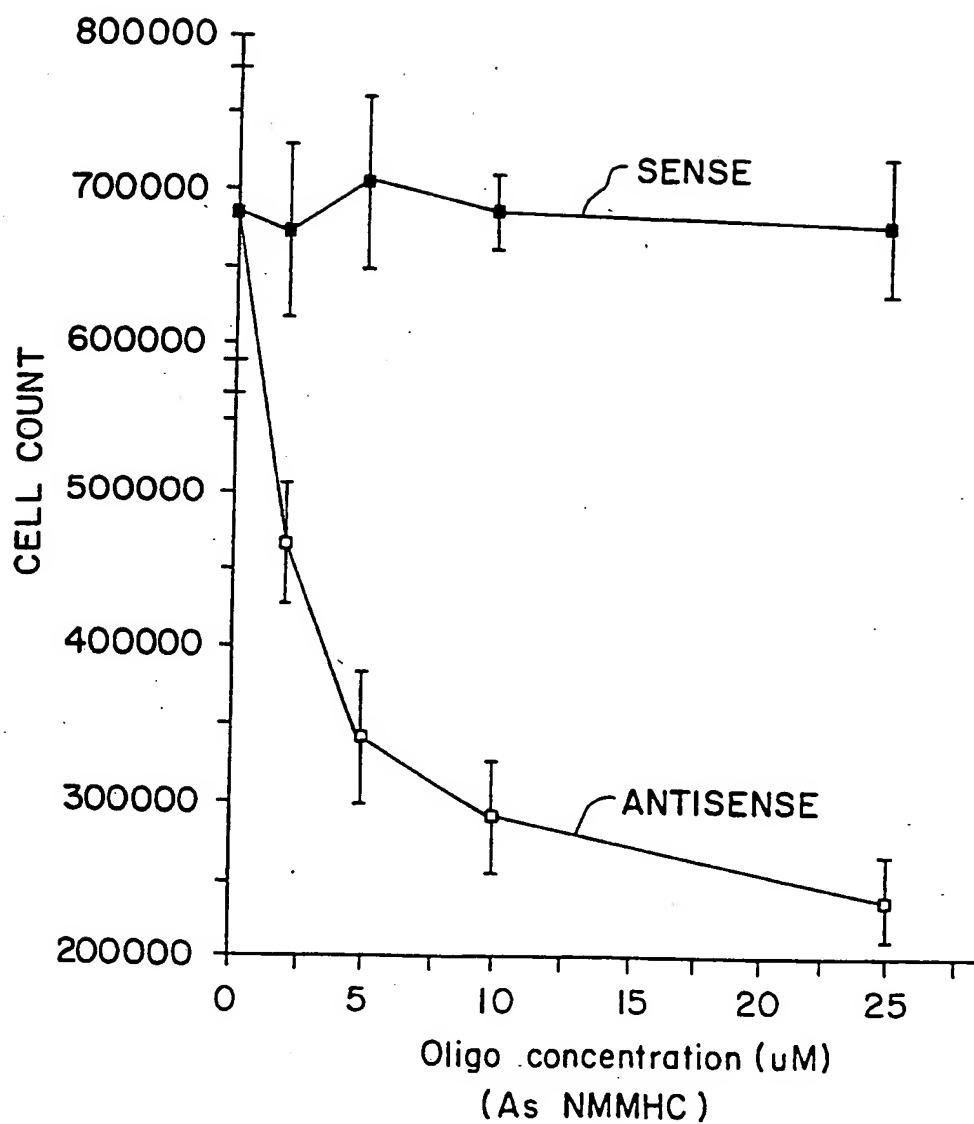
45. The use according to claim 44 wherein said oligonucleotide comprises about 14 to 38 nucleotide bases.

- 49 -

46. The use according to claim 44 wherein said oligonucleotide inhibits translation of the target sequence.

1 / 1 1

FIG. 1A



2 / 1 1

FIG. 1B

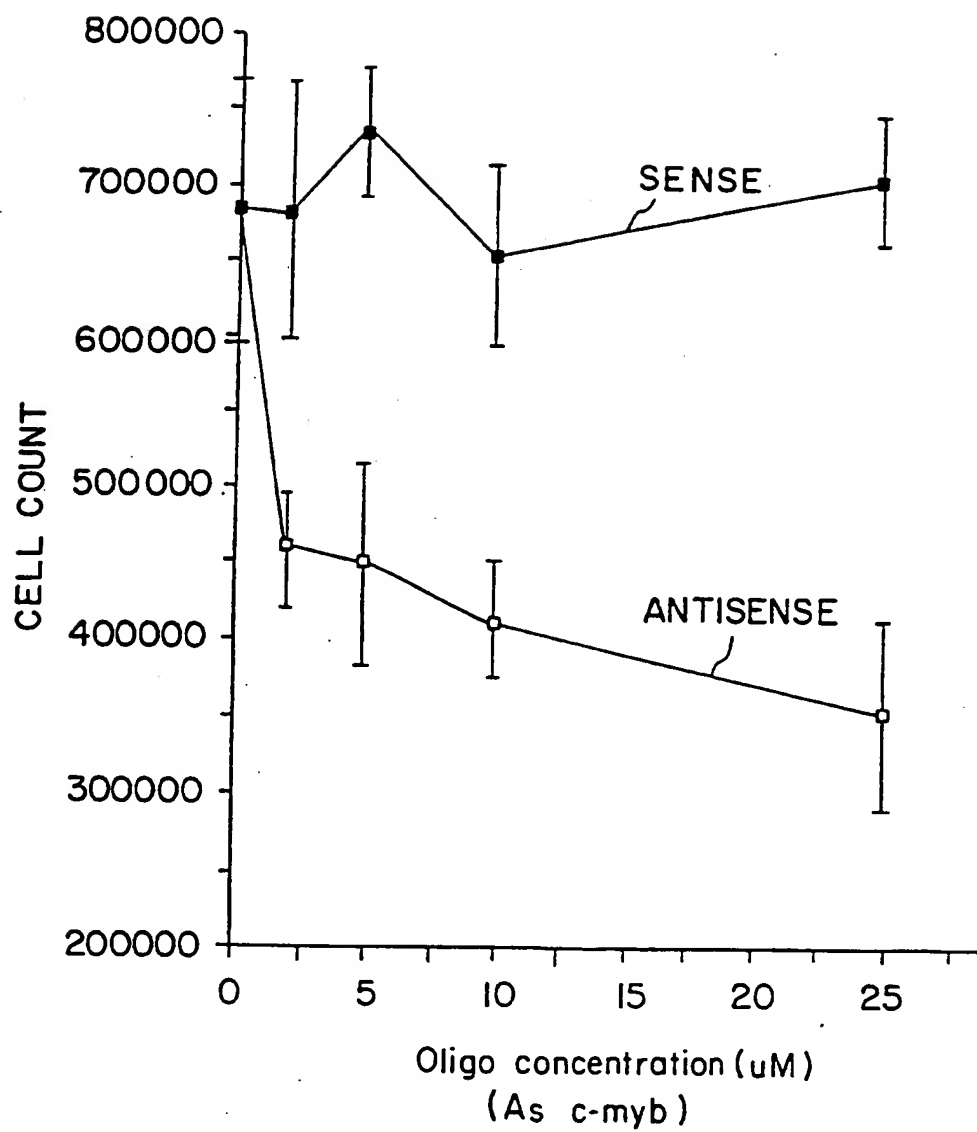


FIG.2A

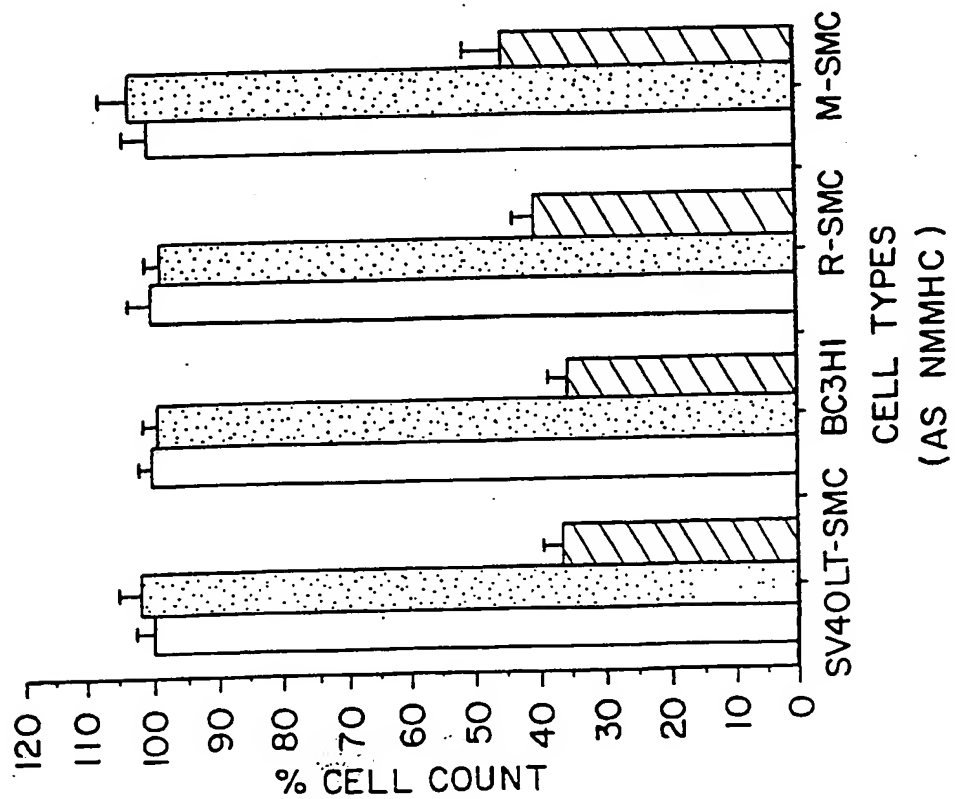
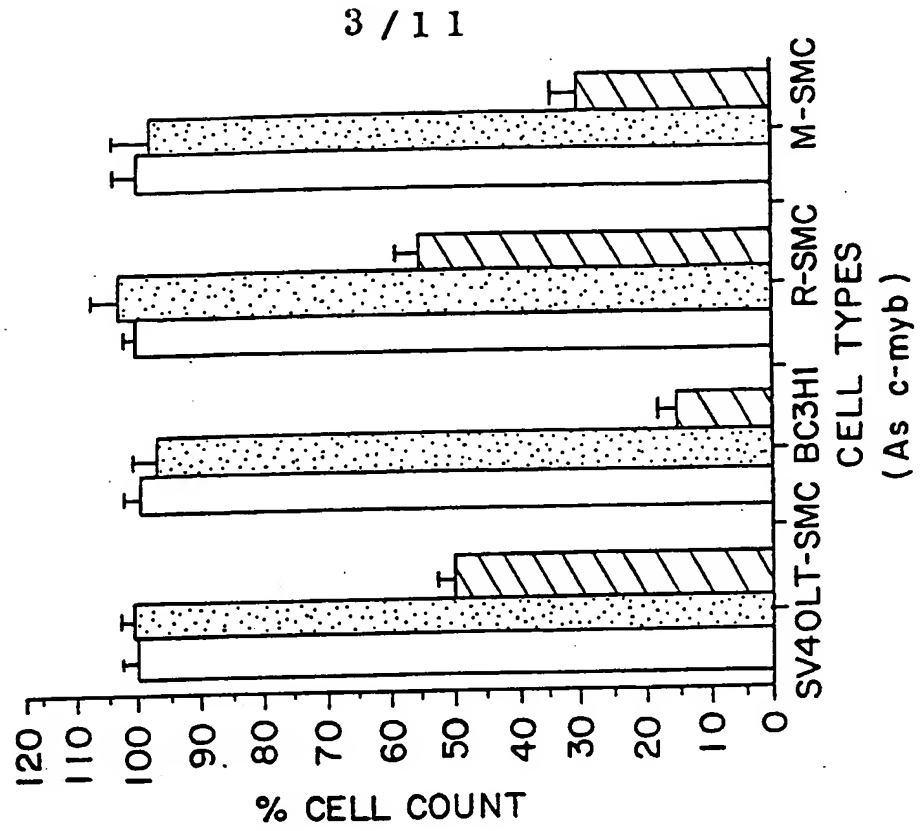
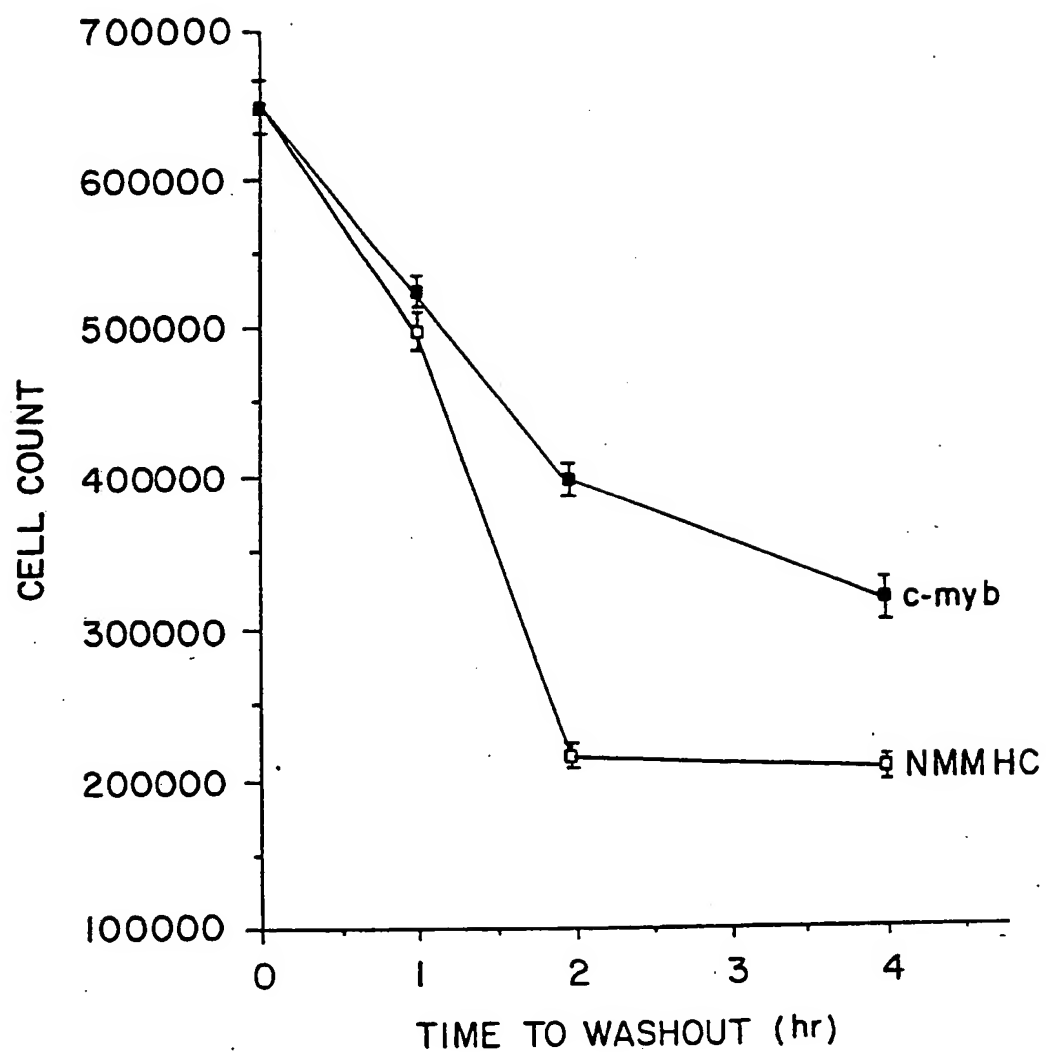


FIG.2B



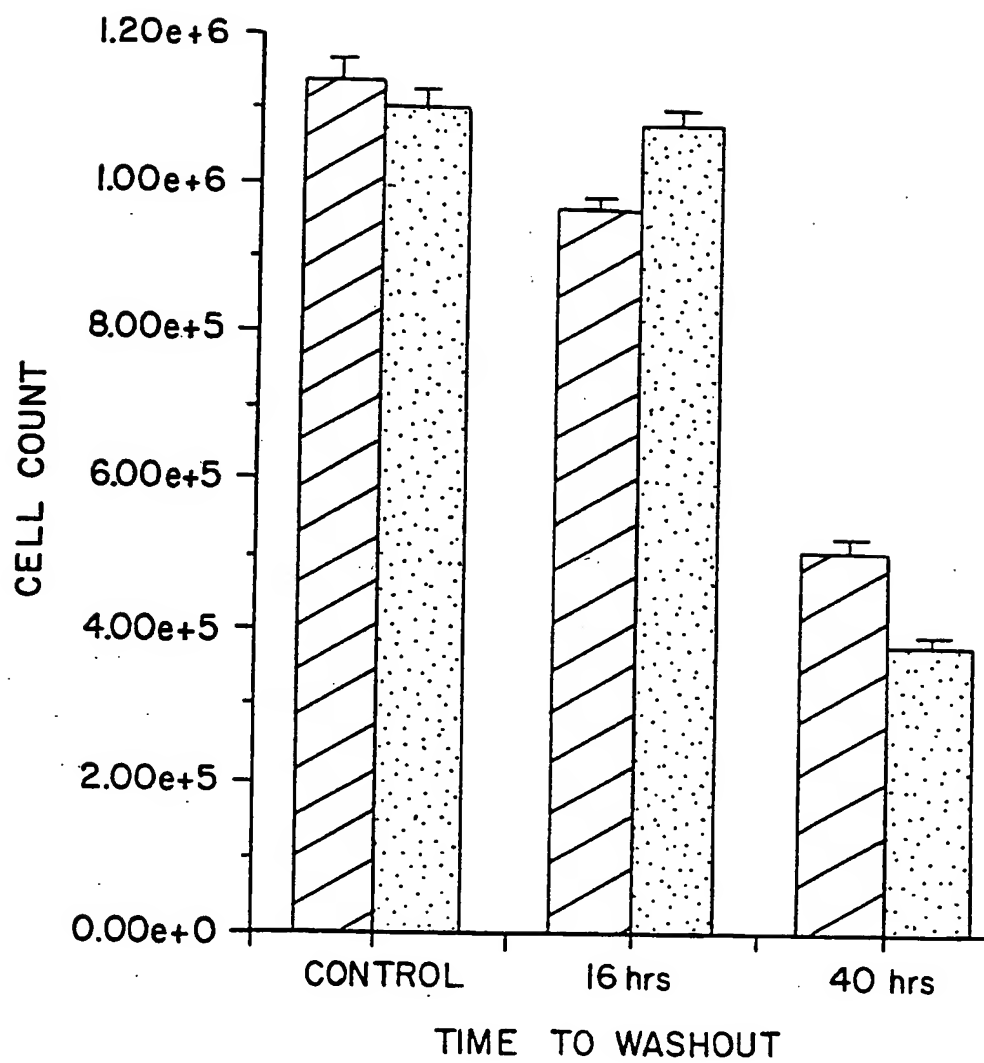
4 / 1 1

FIG. 3A



5 / 1 1

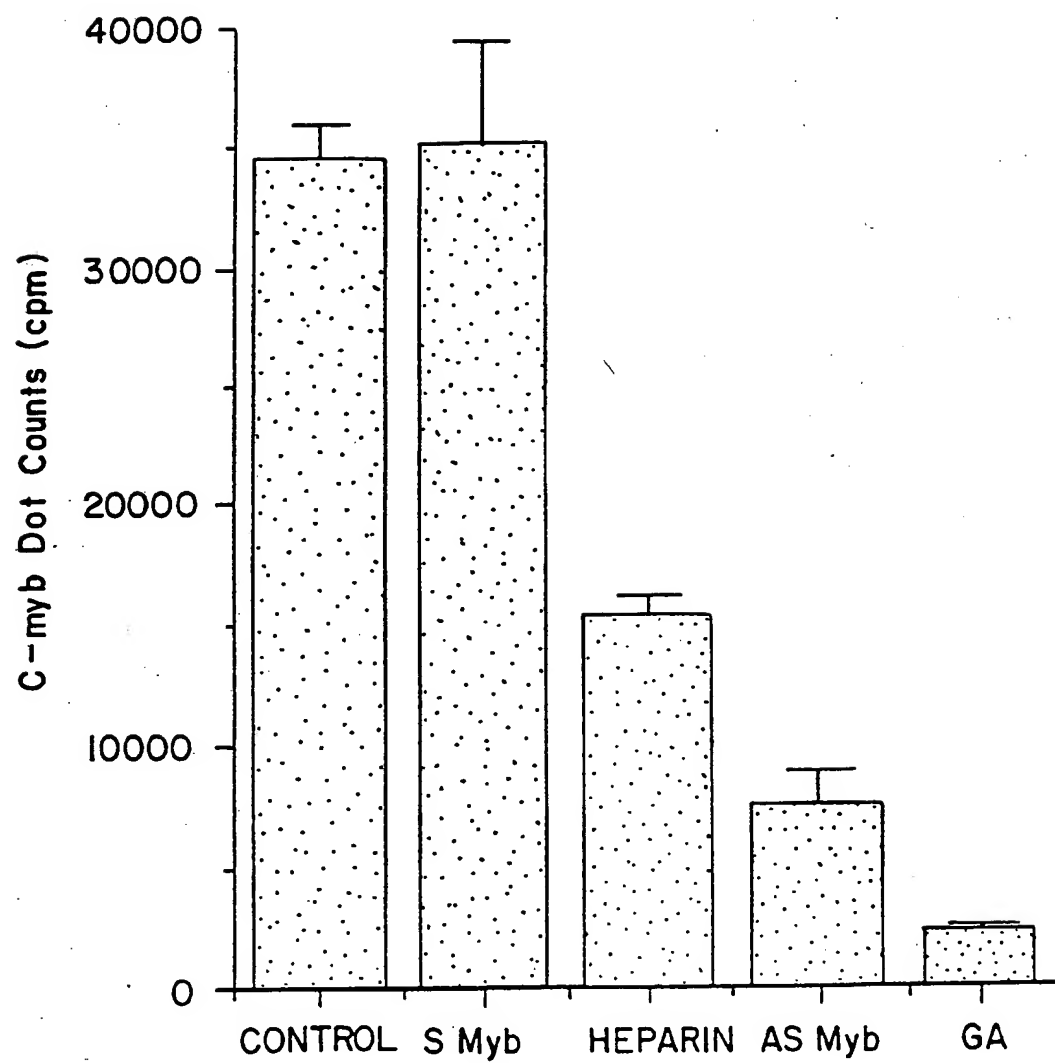
FIG.3B





6 / 1 1

FIG.4



7 / 1 1

FIG. 5

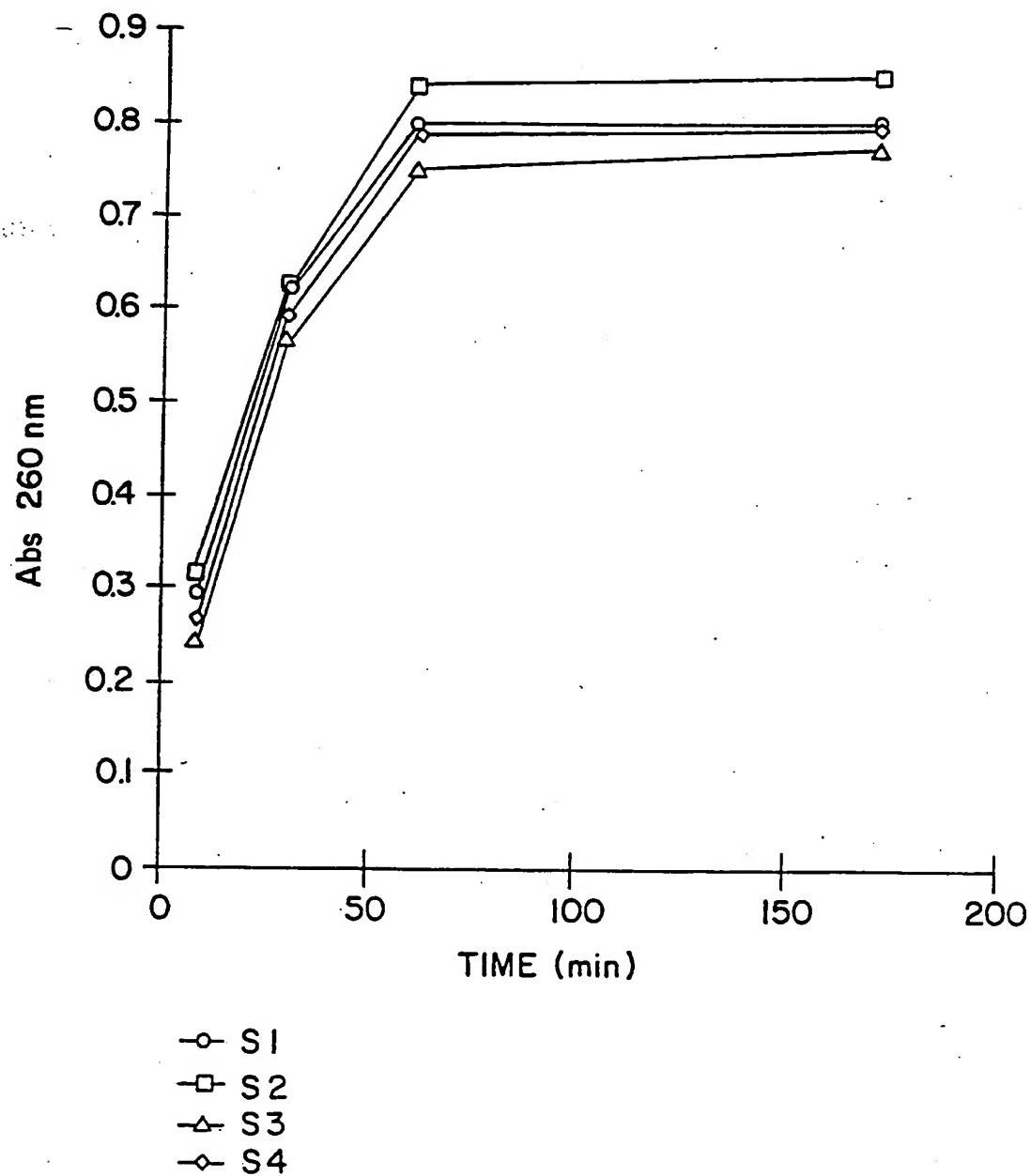


FIG.6

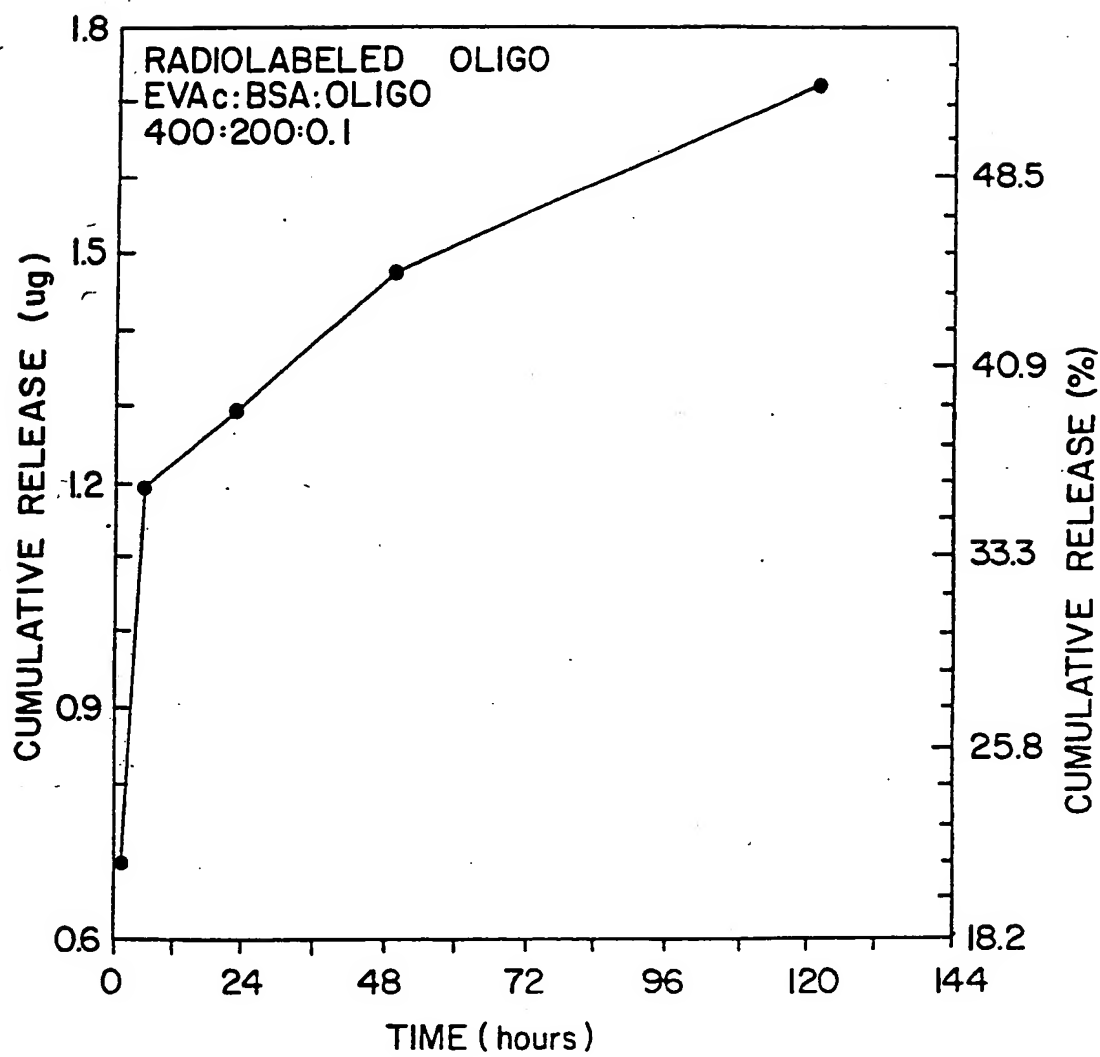
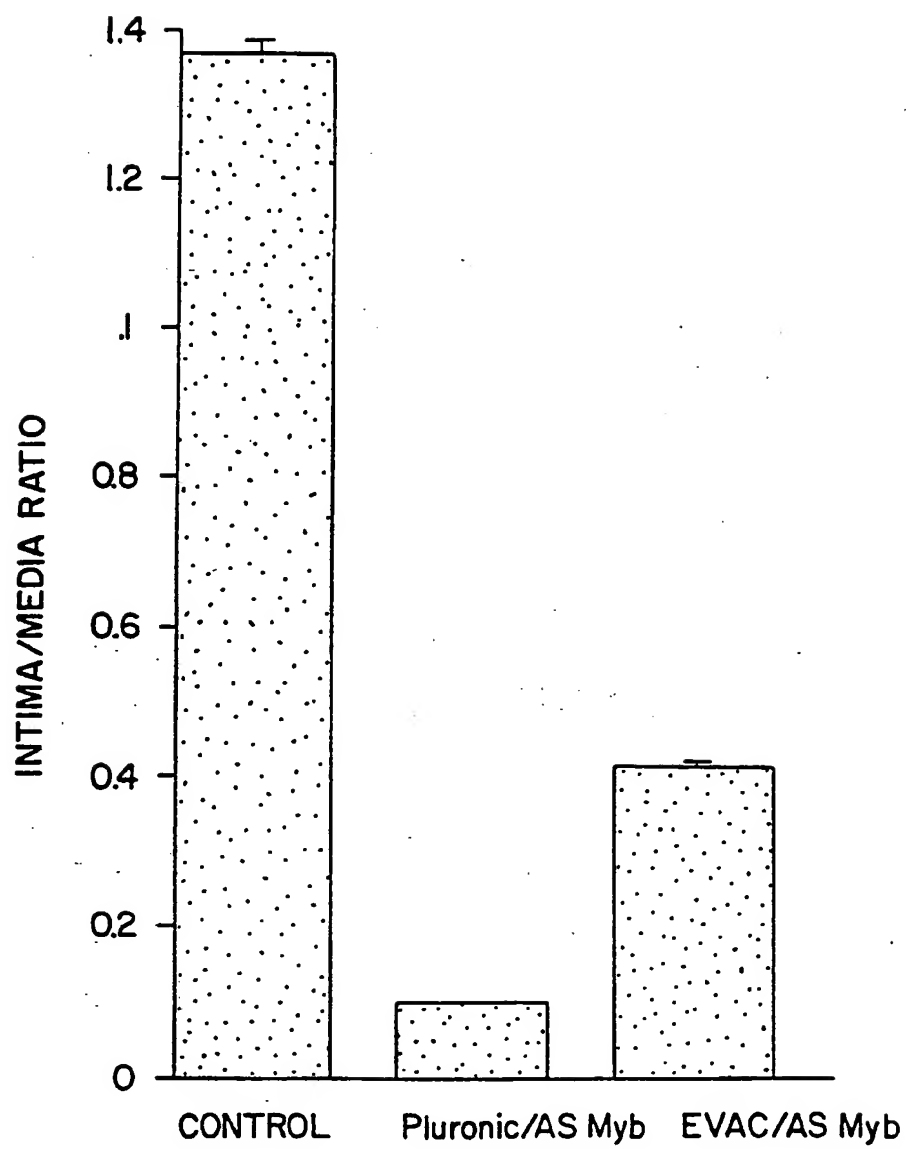
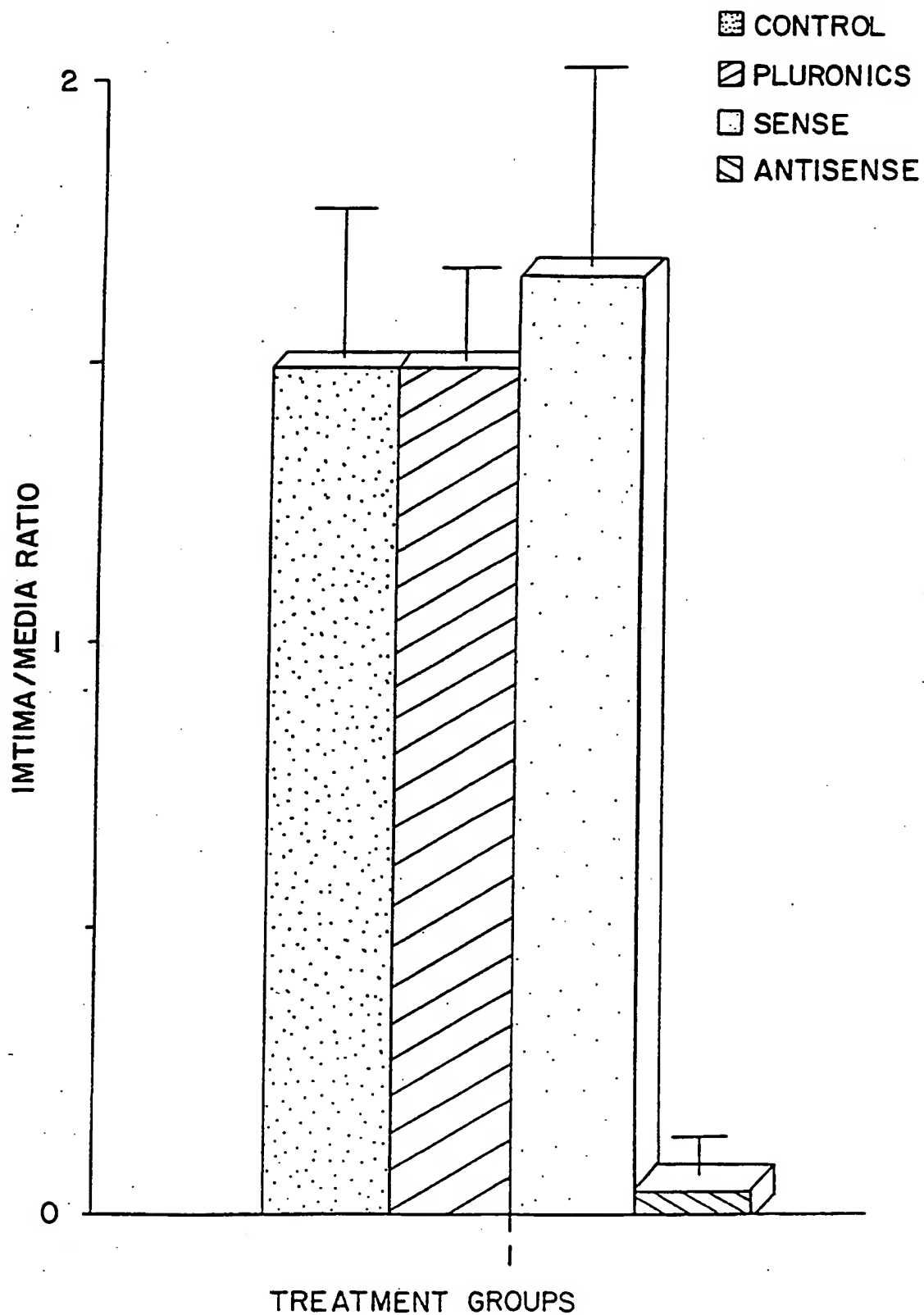


FIG.7



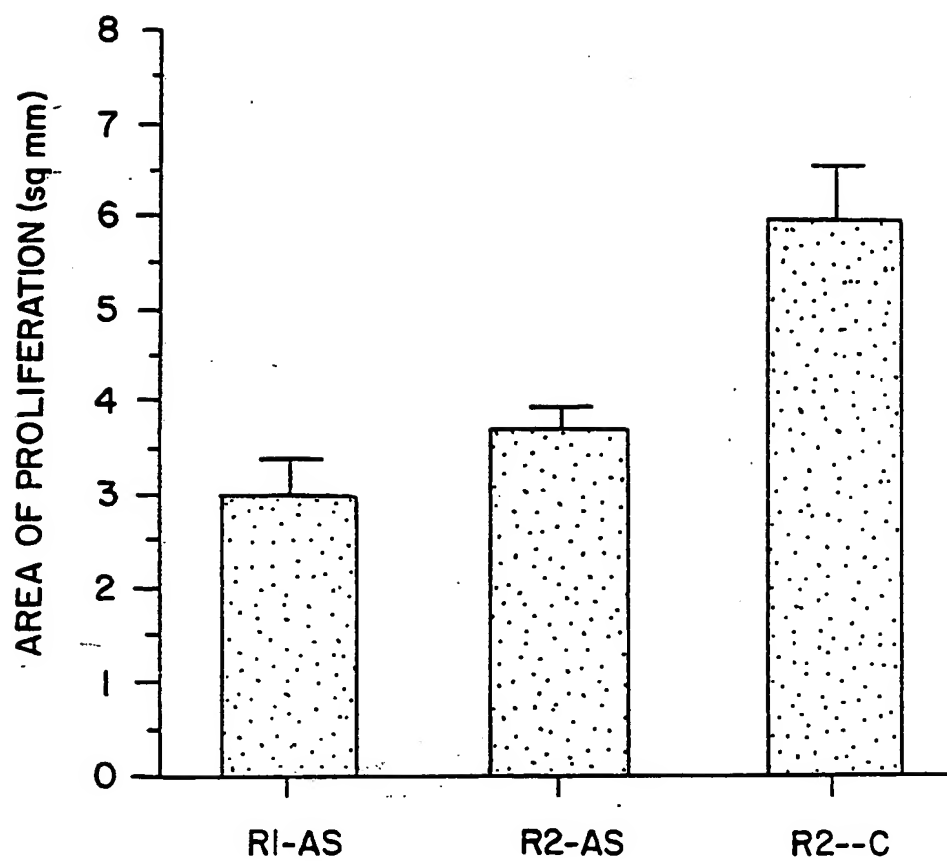
10/11

FIG.8



11/11

FIG.9



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/09626

## A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI, CLAIMS, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9115226 (THE AMERICAN NATIONAL RED CROSS), 17 October 1991 (17.10.91), see page 20, line 21 - page 23, line 10 and the whole document	30-46
A	US, A, 4806463 (JOHN GOODCHILD ET AL.), 21 February 1989 (21.02.89)	30-46
A	US, A, 4740463 (ROBERT A. WEINBERG ET AL.), 26 April 1988 (26.04.88)	30-46

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*B\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*Z\* document member of the same patent family


Date of the actual completion of the international search

16 March 1993

Date of mailing of the international search report

02.04.93

Name and mailing address of the ISA/



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Authorized officer

JONNY BRUN

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/09626

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9103260 (TEMPLE UNIVERSITY OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION), 21 March 1991 (21.03.91)	30-46
A	WO, A1, 9112811 (ISIS PHARMACEUTICALS, INC.), 5 Sept 1991 (05.09.91)	30-46



**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-29  
because they relate to subject matter not required to be searched by this Authority, namely:  
pls. see Rule 39.1(iv)-PCT: Method for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

26/02/93

International application No.

PCT/US 92/09626

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9115226	17/10/91	AU-A- 7676191 EP-A- 0527790	30/10/91 24/02/93
US-A- 4806463	21/02/89	AU-A- 7487787 EP-A- 0412964 WO-A- 8707300	22/12/87 20/02/91 03/12/87
US-A- 4740463	26/04/88	NONE	
WO-A1- 9103260	21/03/91	AU-A- 6410790 CA-A- 2065294 EP-A- 0489846	08/04/91 02/03/91 17/06/92
WO-A1- 9112811	05/09/91	AU-A- 7747091 EP-A- 0517859	18/09/91 16/12/92